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13. ABSTRACT (Maximum 200 Words) <p>Recently, coactivator and corepressor complexes required for function of estrogen receptor (ER), and other nuclear receptors, have been identified, with critical implications for aspects of etiology and therapy of breast cancer. Studies of the mechanisms of estrogen receptor and tamoxifen actions in ours and in other laboratories have permitted new insights to breast cancer diagnosis and therapy.</p> <p>Previous results have shown that the most widely utilized anti-estrogen in the treatment of breast cancer, Tamoxifen causes ER to associate with the N-CoR corepressor complex, and that this association is required for its anti-estrogen effects. Inhibition of N-CoR binding actually causes a switch in tamoxifen function from inhibition to activation. Thus, we hypothesize that resistance in ER-positive tumors is frequently based on alterations in levels or on post-transcriptional modifications in N-CoR, or other members of the corepressor complex that will abolish recruitment of the corepressor complex to the receptor resulting in a "switch" from antagonist to agonist actions. I propose to test this hypothesis using a genetic approach.</p> <p>My experiments will determine the levels of expression of N-CoR protein and mRNA during normal mammary gland development, as well as to determine the cellular localization patten of N-CoR with respect to ER during development. Next, two genetic approaches will be utilized to test the biological role of N-CoR during normal development. First, analysis of mammary glands of mice in which N-CoR has been deleted will be performed over the course of normal mouse mammary development. Tissues from N-CoR gene-deleted mice will be treated with the carcinogen DMBA to determine if deletion or expression of N-CoR enhances or suppresses mammary tumors. Our studies should provide novel insight into the biological role of N-CoR during normal mammary development and its potential to function as a tumor suppressor and in drug resistance.</p>				
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INTRODUCTION

Estrogen stimulates ductal morphogenesis and lobuloalveolar proliferation *in vivo* in the normal breast (reviewed in 1) as well as to stimulate proliferation of estrogen receptor (ER)-positive breast cancer cell lines like MCF-7. While cell lines have provided suitable models for the study of ER mouse genetic models provide a more natural environment in which to investigate the normal physiology of the breast and to identify the molecular alterations that occur during the process of tumorigenesis. The experiments described in my proposal were designed to elucidate the physiological contributions of N-CoR to proliferation, differentiation, and physiology as well as the molecular mechanisms of ER-mediated gene activation and repression.

Classical hormone ablation experiments have demonstrated that the steroid hormone estrogen (E) and progesterone (P) are required for ductal morphogenesis during puberty and proliferation of alveoli during pregnancy (1). Estrogenic compounds can activate either of two receptors; the classical ER α and the more recently discovered ER β . ER β shares 95% and 55% homology with DNA-binding and ligand binding domains of ER α , respectively (2). Deletion of estrogen receptor alpha (ER α), the predominant form of ER expressed in the mammary gland, has shed insight into its role during mammary gland development (3). Deletion of ER α results in a failure of the ductal epithelium to penetrate the stromal fat pad during ductal morphogenesis as a result of defects residing within the stromal component (4).

The development of inhibitory ligands for the nuclear receptors yielded important therapeutic treatments, among them the use of the anti-estrogen tamoxifen for endocrine therapy of breast cancer. The tamoxifen-related compounds, including trans-hydroxytamoxifen (TOT) are thought to inhibit estradiol-dependent transactivation by competitive binding to ER (reviewed in 5,6). However, in certain tissues such as uterus and bone, and in patients in long term treatment with breast cancer, tamoxifen exhibits partial agonistic activity, thought to be mediated by the constitutively active activation function 1 (AF1) domain of ER. However, the molecular mechanism by which tamoxifen exerts differential effects in various tissues has remained elusive.

The cloning and biochemical characterization of a protein which functions as a repressor of unliganded thyroid hormone receptor (TR) and retinoic acid receptors (RAR), nuclear receptor corepressor (N-CoR), has led to insights into the molecular mechanisms of nuclear receptor-mediated gene regulation (7,8). Ligand binding causes decreased interaction of N-CoR to TR and RAR on most DNA sites in both biochemical assays (6,7) and intact cells (9). The Rosenfeld laboratory demonstrated that, upon the binding of the anti-estrogen TOT, ER binds to N-CoR effectively. However, in the absence of N-CoR, TOT is converted from an antagonist to an agonist with regards to ER function (10). The ability of N-CoR to function as a repressor of ER-mediated transactivation suggests that N-CoR may play an important role not only during normal mammary gland biology, but also in the process of breast tumorigenesis and the acquired resistance to tamoxifen treatment. To test the hypothesis that N-CoR is a critical component of the repression complex which mediates tamoxifen's ability to act as an inhibitor of ER, I suggested testing these event in a genetic system which could show which ER is activated by agonists in the absence of the N-CoR complexes. Furthermore, it is possible that tamoxifen resistance in breast cancer patients results from either decreased levels of N-CoR expression or through inhibition of N-CoR's interaction with ER so that the co-repressor cannot be effectively recruited.

Using the single cell nuclear microinjection assay with affinity-purified specific anti-NCoR IgG, our experiments have documented that, in the absence of N-CoR, TOT is converted from antagonist to agonist function. This activation was dependent upon the N-terminal (AF1) domain of ER. Additionally, the interaction of N-CoR with ER is impaired in cells in which tamoxifen acts as an activator and in breast tumors derived from MCF-7 cells implanted into athymic nude mice that develop tamoxifen resistance (10). Furthermore, western blot analysis performed on whole cell extracts from these tumors showed that N-CoR levels decrease in tumors that have developed resistance to the antiproliferative effect

of TOT. These data suggest that either a decreasing level of N-CoR or the inhibition of the co-repressor binding to the receptor, might explain the ability of tamoxifen to induce ER activation in specific cell types, as well as in late stages of breast cancer. Indeed, this hypothesis has been confirmed using cells from an N-CoR gene-deleted mouse model (11). Finally, my preliminary results examining N-CoR levels in normal mouse mammary glands show that N-CoR mRNA levels increase during pregnancy, peaking at or around lactation and declining thereafter, indicating a role for N-CoR in normal mammary gland development. The expression of SMRT follows a similar expression profile.

BODY:

Specific Aims: I have pursued two Specific Aims in my proposal. To thoroughly examine the expression of N-CoR mRNA and protein during the full course of mammary development and to determine the localization of N-CoR protein in relationship to ER-alpha (ER α) during the course of normal development, and to emulate the biological roles of N-CoR in breast development and tumorigenesis. Several areas have been evaluated.

Regulation of N-CoR Function. Understanding the regulation of N-CoR and its associated corepressors, in concert with defining the molecular mechanisms that underlie regulation by androgen receptor, offers a reasonable prospect of achieving new approaches to diagnosis and treatment of prostate cancer. Using the genetic model of an N-CoR (γ -) mouse, we have been able to establish that androgen receptor antagonists require the actions of N-CoR, in a fashion analogous to the actions of estrogen receptor antagonists, without N-CoR, they can function as full agonists. This surprising result indicates that any mechanism that permits escape from the actions of N-CoR will be effective in generating "resistance" to antagonists.

Yeast two hybrid screens were performed using each of the N-CoR-transcription repressor domains which led to several unexpected observations: The first was the identification of a protein that we refer to as N-CoR associated protein 2 (NAP2), a 70 kD protein that can be present in both cytoplasm and nucleus. This protein binds strongly to N-CoR, and can be immunoprecipitated with α -N-CoR IgG from prostate and other prostate cell lines at endogenous levels of both proteins. GST pull-down assay established that the C-terminus of NAP2 interact best with Repression Domains 1 and 3 of N-CoR. Cotransfection of N-CoR and NAP2 resulted in a striking increase in T3 receptor repressor activity, indicating a corepressor role for NAP2. A specific IgG against NAP2 marked by recording the expected 70 kDa protein on tissue Western blot analysis, presented us the opportunity to explore the potential regulators of NAP2.

We find that NAP2 is predominantly nuclear in localization in quiescent cultures, but can be regulated to exhibit virtually complete cytoplasmic localization. One of the first pathways we have identified is regulation of TAB2 by TNF α /IL-1. Addition of IL-1 to cell cultures causes a progressive, dramatic relocation of NAP2 from nucleus to cytoplasm which is blocked by leptomycin B, indicating regulation at the level of nuclear export. The C-terminus of TAB2 protein, which itself exhibits similar responses to IL-1, contains a classical nuclear export signal. Mutation of this motif blocks IL-1 dependent relocation from nucleus to cytoplasm. Therefore, given its association with N-CoR, we investigated whether IL-1 also induced nuclear \rightarrow cytoplasmic relocation of N-CoR. Over a period of \sim 1 hr, most of the N-CoR was now found to be cytoplasmic. Thus, export commences by several minutes, but translocation of N-CoR continues over 60-90 min, with dramatic redistribution evident by 30-60 min.

Because of the presence of a consensus nuclear export signal in NAP2, we mutated four residues, which now eliminated the ability of IL-1/TNF α to cause translocation of NAP2 from nucleus to cytoplasm. Therefore, we tested whether expression of NAP2 containing the mutated nuclear export signal (NES) (NAP2 NES mut) would inhibit N-CoR translocation in response to IL-1/TNF α , finding that it was itself not translocated and blocked IL-1 dependent translocation of N-CoR. Therefore, NAP2 represents an intriguing candidate for one component of regulation of N-CoR function. We and others have established that the

highest affinity N-CoR/SMRT complex contains HDAC3 (12,13). I will now evaluate whether the Tbl1 factor present in an N-CoR/HDAC3 complex (12) is in the same or distinct complex with NAP2. I will use specific antibodies we raised against Tbl1 and NAP2 (12) cell lines expressing NAP2 tagged with both multiple Myc and HA epitopes and use the two step affinity purification. The NAP2/N-CoR complex appears to contain HDAC3, but not HDAC1, HDAC2, or mSin3, so the HDAC3/NAP2 high-affinity complex might be crucial for NAP2 actions.

In parallel, single cell nuclear microinjection assays (11) were performed, initially employing affinity-purified anti NAP2 IgG, revealing that N-CoR now fails to relocate from nucleus to cytoplasm in response to an IL-1 signal. We can conclude that NAP2 is required for IL-1-induced redistribution of N-CoR. Similarly, the loss of specific HDACs could be linked to NAP2, and our initial experiments will emphasize HDAC1, HDAC2, HDAC3, HDAC4, and HDAC5. How does IL-1 signaling regulate NAP2? We hypothesize that NAP2 it is a target of IL-1-induced activation of MEKK-1. While NAP2 contains >18 potential consensus sequences for MEKK1 phosphorylation, the NAP2 C-terminal region that is sufficient to exhibit regulated translocation and interaction with N-CoR contains 8 potential sites flanking the NES. Every potential phosphorylation site (consensus S→A) was mutated and assayed for the ability to exhibit IL-1/TNF α induced nuclear-cytoplasmic translocation. Our preliminary data suggest a single site (aa 419-423), N-terminal of the export signal, may exert a specific regulatory role. This would imply that NAP2 is capable of interacting with N-CoR both in the phosphorylated and unphosphorylated state, but that a conformational change, dependent upon phosphorylation of a specific C-terminal residues serves to initiate a conformational alteration that exposes the export signal. In this case, overexpression of NAP2 harboring a mutation of this regulatory phosphorylation site should serve as a dominant-negative regulator of IL-1-dependent N-CoR relocalization. Because there appears to be a high affinity interaction between NAP2 and N-CoR, we tested the ability of NAP2 to directly interact with HDAC3 by GST-pull down assay and by immunoprecipitation from LNCaP, 293, Rat-1 cells in the presence and absence of IL-1 or TNF α . Recently, we have found that interaction of the SANT domain in N-CoR with HDAC3 increases its histone deacetylase (13). Thus, the actions of NAP2 on enzymatic function of HDAC3 alone and in concert with N-CoR, will be assessed using coimmunoprecipitation assays, and using biochemical approaches. Using Anti-MEKK1 IgG, the effects on IL-1 β induced translocation could be assessed in single cell assays. We found that α MEKK1 IgG blocked IL-1 β -induced translocation to cytoplasm.

The next issue was to explore the effects of the IL-1/TNF α pathways on actions of specific classes of DNA binding transcription factors. Based on the relationship of the TNF α /IL-1 pathway to activation of NF κ B, we will explore NF κ B-regulated genes. In particular, transcription units are described to bind either the p65/p50 heterodimers or p50 homodimers which are suggested to repress gene expression. The search for IL-1 responsive genes has revealed that these include a metastasis suppressor gene, as well as genes such as ICAM1 and IL-6. To address this further, we investigated potential downstream target genes using the chromatin immunoprecipitation (ChIP) assay. The KAI1 gene was chosen for study because it has been reported to be a metastasis suppressor gene for prostate cancer, and possibly also for breast and lung cancer (14). KAI1 encodes the membrane tetraspanin that is linked to cell adhesion interactions with transmembrane helix receptors, and growth factor receptors. Metastasis, the leading cause of death for most cancer patients, remains one of the least understood aspects of prostate cancer biology. Decreased expression of the human KAI1 gene is involved in the progression of the cancer, and the KAI1 gene appears to be regulated by signaling molecules that activate this NF κ B target gene. We found that the p50 component of NF κ B did not directly interact with NAP2 but was found to exhibit N-CoR-dependent binding of NAP2. A chromatin immunoprecipitation assay (ChIP) was used to evaluate the presence of p50, p65, NAP2, and N-CoR on the KAI1 promoter. In the absence of treatment, p50, but not the p65 NF κ B subunit, as well N-CoR and NAP2 were present.

After IL-1 treatment, the NAP2 and N-CoR were selectively lost, and immunohistochemistry revealed that NAP2 and N-CoR were exported from the nucleus. These data indicate that N-CoR/NAP2 might be involved in the regulation of the expression of an important metastasis suppressor gene. Therefore, a metastasis suppressor gene is a potential target gene repressed by p50 homodimers. To test whether the presence of NAP2 causes active repression, we will use NAP2 IgG and a KAI-1/LacZ from reporter gene expression, in the single cell nuclear microinjection assay (11). These studies revealed that export from nucleus is blocked by α MEKK1. However, activation required recruitment of specific coactivators including the MYST HAT, Tip60.

Role of the SMRT Corepressor. Finally, because N-CoR and SMRT exhibit complimentary activity in many actions, we have generated SMRT^{-/-} mice, and will investigate the effects on androgen and estrogen receptor actions, eventually using MEFs prepared from SMRT^{-/-} and N-CoR^{-/-}/SMRT^{-/-} embryos. In these experiments, we will utilize single cell nuclear microinjection assays, as previously described (11) with specific reporters, to determine activity of agonist and antagonists. We are analyzing prostate development in SMRT^{-/-} mice, N-CoR^{-/-}, and SMRT^{-/-} are being generated as these mice are viable.

Key Research Accomplishments:

- Generation of N-CoR-specific antibodies and probes.
- Temporal/Spatial pattern of N-CoR expression defined.
- Generation of mice deleted for the related gene SMRT.
- Initial analysis of SMRT expression during development.
- Generation of MEF lines from wild type and N-CoR^{-/-} mutant mice.
- Demonstration that estrogen receptor recruits at least two distinct N-CoR-containing complexes as mSin/HDAC 2/1 and also the novel TAB2/HDAC3/N-CoR complex that bind to antagonist.
- Identification of a novel N-CoR complex containing a factor TAB2 and HDAC3 as a nuclear repressor complex.
- Evidence that inflammatory signals such as IL-1 β causes export of the TAB2/N-CoR/HDAC3 complex from nucleus to cytoplasm.
- Evidence that IL-1 β activates MEKK1, which phosphorylates TAB2, causing exposure of nuclear export signals and permitting the N-CoR/TAB2/HDAC complex to be exported.
- Evidence that IL-1 β with export of the TAB2/N-CoR/HDAC complex causes estrogen antagonists to function as full agonists.

Conclusions: We can conclude that clinically appropriate response to estrogen antagonists can be linked to activation on binding of an N-CoR/HDAC2/TAB2 complex, required to prevent antagonist as agonists. The definition of this pathway could be extended in the coming year and uses to provide export of the complex will be explored. Finally, in these studies, the role of the TSE has been uncovered and I will explore this over the coming year.

Reproducible Outcomes: Abstract: Nuclear Receptor Corepressor Regulation of Estrogen Receptor Antagonist Function. September 25-28: Era of Hope meeting.
Poster Presentation: Transcription, Translation and Modification

References Cited:

1. Daniel, C.W. and G.B. Silberstein. 1987. Postnatal development of the rodent mammary gland. In *The mammary gland* (ed. M.C. Neville and C.W. Daniel), pp. 3-36. Plenum, New York.
2. Kupier, G.G., E. Enmark, M. Peltö-Huikko, S. Nilsson and J.A. Gustafsson. 1996. Cloning of a novel receptor expressed in rat prostate and ovary. *Proc Natl Acad Sci* **93**: 5925-5930.
3. Lubahn, D.B., J.S. Moyer, T.S. Golding, J.F. Couse, K.S. Korach and O. Smithies. 1993. Alteration of reproductive function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene. *Proc Natl Acad Sci* **90**: 11162-11166.
4. Cunha, G.R., P. Young, Y.K. Hom, P.S. Cooke, J.A. Taylor and D.B. Lubahn. 1997. Elucidation of a role of stromal steroid hormone receptors in mammary gland growth and development by tissue recombination experiments. *J Mammary Gland Biol Neoplasia* **2**: 393-402.
5. Katzenellenbogen, J.A. and B.S. Katzenellenbogen. 1996. Nuclear hormone receptors; ligand-activated regulators of transcription and diverse cell responses. *Chem Biol* **3**: 529-536.
6. Osborne, C.K., E.B. Coronado-Heinsohn, S.G. Hilsenbeck, B.L. McCue, A.E. Wakeling, A. McClelland, D.L. Manning and R.I. Nicholson. 1995. Comparison of the effects of a pure steroidal anti-estrogen with those of tamoxifen in a model of human breast cancer. *J Natl Cancer Inst* **87**: 746-750.
7. Horlein, A.J., A. M. Naar, T. Heinzel, J. Torchia, B. Gloss, R. Kurokawa, A. Ryan, Y. Kamei, M. Soderstrom and C.K. Glass. 1995. Ligand-independent repression by thyroid hormone receptor mediated by a nuclear receptor co-repressor. *Nature* **377**: 397-404.
8. Kurokawa, R., M. Soderstrom, A. Horlein, S. Halachmi, M. Brown, M.G. Rosenfeld and C.K. Glass. 1995. Polarity-specific activities of retinoic acid receptors determined by a co-repressor. *Nature* **377**: 451-454.
9. Perissi, V., J.S. Dasen, R. Kurokawa, Z. Wang, E. Korzus, D.W. Rose, C.K. Glass and M.G. Rosenfeld. 1999. Factor specific modulation of CREB-binding protein acetyltransferase activity. *Proc Natl Acad Sci* **96**: 3652-3657.
10. Lavinsky, R.M., K. Jepsen, T. Heinzel, J. Torchia, T.-M. Mullen, R. Schiff, A.L. Del-Rio, M. Ricote, S. Ngo, S.G. Hilsenbeck, C.K. Osborne, C. K. Glass, M.G. Rosenfeld and D.W. Rose. 1998. Diverse signaling pathways modulate nuclear receptor recruitment of N-CoR and SMRT complexes. *Proc Natl Acad Sci* **95**: 2920-2925.
11. Jepsen, K., O. Hermanson, T.M. Onami, A.S. Gleiberman, V. Lunyak, R.J. McEvilly, R. Kurokawa, V. Kumar, F. Liu, E. Seto, S.M. Hedrick, G. Mandel, C.K. Glass, D.W. Rose, and M.G. Rosenfeld. 2000. Combinatorial roles of the nuclear receptor co-repressor in transcription and development. *Cell* **102**: 1-20.
12. Guenther, M., Lane WS, Fischle W, Verdin E, Lazar MA, Shiekhhattar R. 2000. A core SMRT corepressor complex containing HDAC3 and TBL1, a WD40-repeat protein linked to deafness. *Genes and Development*. **14**, 1048-1057.
13. Wen, Y., V. Perissi, L. Straszewski, W. Yang, C. Glass, M. Rosenfeld, and E. Seto. 2000. The histone deacetylase-3 complex contains nuclear receptor corepressors. *Proc Natl Acad Sci USA*. **97**, 7202-7207.
14. Dong, Jin-Tang, W.B. Isaacs, J.C. Barrett, and J.T. Isaacs. 1997. Genomic organization of the Human KAI 1 metastasis-suppressor gene. *Genomics* **41**: 25-32.